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## Prostaglandin E2 affects T cell responses through modulation of CD46 expression<sup>1</sup>

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### Abstract

The ubiquitous protein CD46, a regulator of complement activity, promotes T cell activation and differentiation towards a regulatory Tr1-like phenotype. CD46-mediated differentiation pathway is defective in several chronic inflammatory diseases, underlying the importance of CD46 in controlling T cell function and the need to understand its regulatory mechanisms. Using an RNAi-based screening approach in primary T cells, we have identified that two members of the G-protein coupled receptor (GPCR) kinases were involved in regulating CD46 expression at the surface of activated cells. We have investigated the role of prostaglandin E2 (PGE2), which binds to the E-prostanoid family of GPCRs through four subtypes of receptors called EP1-4, in the regulation of CD46 expression and function. Conflicting roles of PGE2 in T cell functions have been reported, and the reasons for these apparent discrepancies are not well understood. We show that addition of PGE2 strongly downregulates CD46 expression in activated T cells. Moreover, PGE2 differentially affects T cell activation, cytokine production and phenotype depending on the activation signals received by the T cells. This was correlated with a distinct pattern of the PGE2 receptors induced, with EP4 being preferentially induced by CD46 activation. Indeed, addition of an EP4 antagonist could reverse the effects observed on cytokine production observed following CD46 costimulation. These data demonstrate a novel role of the PGE2-EP4-GRK axis in CD46 functions, which might at least partly explain the diverse roles of PGE2 in T cell functions.

### INTRODUCTION

CD46 is a ubiquitously expressed type I membrane protein, that was first identified as a regulator of the complement cascade, preventing autolysis of cells by binding to C3b/C4b and allowing their cleavage by protease I (1, 2). About 10 years ago, CD46 was shown to link innate immunity to acquired immunity. Indeed, costimulation of the TCR with CD46 leads to increased T cell proliferation (3), and affects T cell morphology (4) and polarity (5). Importantly, CD46 also drives Tr1 differentiation, characterized by secretion of high

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amounts of IL-10 (6) and granzyme B (7). IL-2 is key in CD46-mediated Tr1 differentiation, acting as a sensor to switch T cells from a Th1 to a Tr1 phenotype (8). The enzymatic processing of CD46 is a crucial feature of CD46-mediated pathway that is involved in regulating T cell function. CD46 surface expression is strongly downregulated upon its own triggering, partly due to MMP cleavage of its ectodomain (9–11). This is followed by cleavage by gamma-secretase of the two cytoplasmic tails of CD46, which is important to initiate and terminate T cell responses (11, 12). This again underlines the importance of the plasticity of CD46 in controlling T cell homeostasis. Moreover, CD46-mediated Tr1 differentiation is altered in patients with multiple sclerosis (MS), characterized by an impaired IL-10 secretion upon CD3/CD46 costimulation (13–16), and the dysregulation of CD46 pathways in T cells was recently described in patients with asthma (17) and in a small group of patients with rheumatoid arthritis (8). The identification of a dysfunctional CD46 pathway in chronic inflammatory diseases highlights its importance in controlling T cell homeostasis, and further underlines the need to understand its regulation and the molecular mechanisms responsible for its functions.

Using an RNAi-based approach (18) to dissect the molecular pathways that regulate CD46 expression on primary human T cells, we identified two members of the serine/threonine kinase GRK (G-protein coupled receptor kinase) family involved in the regulation of CD46 expression. GRKs phosphorylate agonist-activated G-protein coupled receptors (GPCR) (19, 20), resulting in their binding to  $\beta$ -arrestins and subsequent signaling impairment and internalization, a process known as desensitization (21, 22). There are 7 types of GRK referred to as GRK1–7, each with different expression profiles (21). Among them, GRK2, 3, 5 and 6 are ubiquitously expressed, but are expressed at particularly high levels in immune cells, and have been shown to regulate inflammation (23).

Herein, we show that the knockdown of GRK2 and GRK3 strongly decreased CD46 expression, and that activation of CD46 increased GRK2/3 expression levels. GRK2/3 have been shown to regulate prostaglandin E2 (PGE2) receptors, among other GPCRs (24). As PGE2 is a known modulator of T cell functions (25), we assessed the role of PGE2 in the regulation of CD46 expression and function, in order to demonstrate a role of GRKs in the CD46 pathway. PGE2 notably inhibits T cell proliferation by downregulating both IL-2 and the IL-2R $\alpha$  chain (CD25) (26). PGE2 can also markedly reduce production of Th1 associated cytokines such as IFN $\gamma$ , causing a switch from a Th1 to a Th2 cytokine secretion profile in these cells (26, 27). However, PGE2 has also been shown to promote Th1 differentiation (28, 29), and to either decrease (30, 31) or promote IL-17 production (32–35). PGE2 can also induce Foxp3 in naive CD4<sup>+</sup> T cells, with an increase in regulatory cell function (36). Hence, multiple effects of PGE2 have been reported, and although the local concentrations of PGE2 are important to control T cell differentiation (28), the reasons for these apparent discrepancies are not well understood. Moreover, there are no studies on any potential effects of PGE2 on the CD46-mediated pathway. Herein, we first demonstrate that the addition of PGE2 to T cell cultures strongly decreased CD46 expression in activated T cells. Second, we show that the addition of PGE2 differentially affected T cell responses depending on the activation signals, as responses to CD28 and CD46 costimulation were different. While T cell proliferation was decreased in all conditions, the IL 10:IFN $\gamma$  ratio was either increased or decreased depending on the activation status. Moreover, specific changes in the phenotype of activated T cells were observed. Prostaglandin E2 (PGE2) binds to the E-prostanoid family of GPCRs, through four subtypes of receptors called EP1–4 (24). We show that CD46 and CD28 costimulation led to different patterns of expression of the two PGE2 receptors, EP2 and EP4, with EP4 being preferentially expressed by CD46 activation. Overall, we demonstrate a novel role of the PGE2-EP4-GRK axis in CD46 functions, and how this influences the effects of PGE2 on human T cell activation.

## Materials and Methods

### Antibodies and reagents used

anti-CD3 (OKT3, 5 $\mu$ g/ml), anti-CD28 (CD28.2, 5 $\mu$ g/ml), anti-CD46 (10  $\mu$ g/ml)(20.6, kindly provided by Dr. Chantal Rabourdin-Combe, France). PGE2 (Sigma; 100 nm unless otherwise stated). Recombinant human IL-2 (Tecin) was added at 10U/ml. The selective EP4 antagonist (ONO-AE2-227, 300 nM) was chemically synthesized by Charnwood Molecular Ltd. (Leicester, UK). The EP2 antagonist (AH6809; 10  $\mu$ M) was purchased from Calbiochem (Nottingham, UK). The antibodies for flow cytometry were as follows: anti-CD46-FITC (clone MEM-258; Biolegend); anti-CD25-APC (clone M-A251), anti-CD69-PE (clone FN50) and anti-CTLA-4-PE (clone BNI3) were purchased from BD Pharmingen. Anti-human Foxp3-APC (clone PCH101; ebioscience) recognized all Foxp3<sup>+</sup> T cells (37). Anti-p-Tyr Stat3 (pY705)-pacific blue was purchased from BD Bioscience. The antibodies used for the GRK2/3 western-blot (rabbit) was purchased from Cell Signaling and used at 1 $\mu$ g/ml (in 5% milk-TBST), followed by addition of anti-rabbit IgG-HRP from Promega (1:10,000).

### Cell purification and activation

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ), from heparinized venous blood from healthy donors obtained after informed consent. Ethical approval was obtained from the Lothian Board Ethics Committee. CD4<sup>+</sup> T cells were negatively isolated using magnetic beads (CD4 isolation kit II, Miltenyi Biotec, Auburn, CA, purification > 90%). T cells (1 $\times$ 10<sup>6</sup> cells/1ml/well) were then cultured in 24-culture wells pre-coated with anti-CD3, anti-CD28, or anti-CD46, in RPMI containing 10% FCS. Exogenous IL-2 (10 U/ml) was added to CD3/CD46 stimulated cells as previously described (6). In some experiments, PGE2, selective EP2 or EP4 antagonists and DMSO or ethanol as vehicle control (similar dilution) were added to the culture.

### The RNAi Consortium (TRC) RNAi library

The complete description of the RNAi Consortium lentiviral RNAi library used in this study has been reported (38), and already used to infect primary T cells (18), and is now commercialized by Sigma-Aldrich. Human genes are targeted with ~5 shRNAs expressed under the control of the U6 Pol III promoter in a lentiviral vector (pLKO.1) vector that also confers puromycin resistance. Plasmid DNA purification and lentiviral production were performed as described (38) and <http://www.broad.mit.edu/rnai/trc/lib>.

### T cell stimulation and infection

CD4<sup>+</sup> T cells (2 $\times$ 10<sup>5</sup> cells/well) were cultured in 96-well plates pre-coated with anti-CD3 (OKT3, 2.5 $\mu$ g/ml), anti-CD28 (2D10, 2.5 $\mu$ g/ml), in presence of human recombinant IL-2, (Tecin (TM), National Cancer Institute, Frederick, Maryland)(20U/ml) for 24 hours before infection. The infection was carried out by centrifugation for 90 min at 2300 rpm at room temperature, in presence of the viral supernatant and polybrene (8  $\mu$ g/ml). After removal of the virus, fresh medium was added. Infected cells were then selected by addition of puromycin (2.5  $\mu$ g/ml) 48 hrs after infection, and the cells were expanded in IL-2 for 10 days. Cells were then restimulated with anti-CD3/CD28 antibodies in the presence of IL-2. At 24 hours post-restimulation, the level of CD46 expression at the cell surface was determined by flow cytometry.

### GRK2/3 detection by western-blot

Purified CD4<sup>+</sup> T cells were activated in 48-well plates pre-coated with anti-CD3, anti-CD46 or anti-CD28 Abs, and cultured for 3 days. Cells were then lysed with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 10 mM NaF, 0.4 mM Na<sub>2</sub>VO<sub>4</sub>). Cell lysates were run on a 8% SDS-PAGE and amounts of GRK2 and GRK3 detected by western-blot using anti-GRK2 antibodies that cross react with GRK3 (Cell Signaling). The membranes were then stripped and reblotted with anti-GAPDH MAb to evaluate protein quantities. Densitometry analyses (with image J) were then performed to calculate the GRK:GAPDH ratio.

### Flow cytometry

The expression level of CD46, CD25, CD69 was assessed by flow cytometry with anti-CD46-FITC, anti-CD69-PE, anti-CD25-APC. The presence of CTLA-4 (PE) and Foxp3 (APC) was determined after permeabilization of the cells with 0.1 % saponin. The relative expression to the staining with the isotype control was calculated by calculating the  $\Delta$ MFI (MFI antibody stained - MFI control antibody). Samples were run on a BD FACScalibur. Phosphorylation of Stat3 was analyzed with an anti-p-Tyr Stat3-pacific blue and ran on a BD LSR Fortessa. All flow cytometry data were analyzed using flowJo (Treestar).

### T cell proliferation

Purified T cells ( $2 \times 10^5$  cells/200 $\mu$ l/well) were then cultured in 96-culture wells pre-coated with anti-CD3, anti-CD28, or anti-CD46, in 10% FCS-RPMI for 72hrs, before addition of 1 $\mu$ Ci of [<sup>3</sup>H]thymidine (Amersham). Proliferation was determined using a Liquid Scintillation Counter (Wallac, Boston, USA).

### ELISA

Cytokine production was determined in cell culture supernatants using ELISA specific for human IL-10 (BD Pharmingen, San Jose, CA) and IFN- $\gamma$  (Endogen, Cambridge, MA). Recombinant hIL-10 (BD Pharmingen) and hIFN $\gamma$  (Endogen) were used as standards.

### EP1-4 PCR

These PCR were performed as previously described (39). Briefly, RNAs from activated T cells were extracted using the RNeasy Mini Kit (Qiagen). RNA samples were reverse-transcribed using VILO (Invitrogen, Paisley, UK) according to the manufacturer's guidelines. RT-PCR analysis of EP1, EP2, EP3 and EP4 was carried out using an ABI Prism 7500 (Applied Biosystems, Warrington, UK). Results are expressed relative to a standard (pooled normal human endometrial tissue cDNA) included in all reactions. Data are represented as mean  $\pm$  SEM.

### Statistical analyses

The groups were analyzed using the Graphpad Prism software. Data were analyzed using the Wilcoxon test. All p-values are two-tails and with a 95% confidence interval.

## RESULTS

### An RNAi screen identifies GRKs as novel regulators of CD46 expression in primary human T cells

In order to gain new insights into the regulation of CD46 expression in primary human T cells, we carried out an RNAi screen using a sub-genomic library targeting ~1,000 genes,

focused on kinases and phosphatases, and containing 3–5 shRNAs per gene, as previously described (18). Our goal was to identify novel regulators of CD46 expression in primary human T cells. The level of CD46 expression at the cell surface of T cells infected with lentivirus particles expressing a distinct and unique shRNA was determined by flow cytometry. Using a statistical Z-score (see (18)) to quantify the deviation of CD46 levels from the mean of all measurements within the same plate, we selected shRNAs that significantly modulated CD46 expression, compared to the cohort of control wells consisting of cells infected with control shRNAs (against non-mammalian reporter genes) (Figure 1A). GRK2 and GRK3, two members of the G-coupled protein receptors kinase family (GRK), decreased CD46 expression once knocked-down (Figure 1A, circled). Two shRNA constructs out of four for GRK2 and two out of three for GRK3 led to a similar phenotype (Figure 1A). These data were reproduced in two independent experiments performed with different donors (Figure 1B). Representative plots showing normal CD46 expression compared to expression upon GRK2 and GRK3 knock-downs are shown in Figure 1C. Hence, downregulation of GRKs decreased CD46 expression in activated primary human T cells.

### CD46 activation led to enhanced GRK2/3 levels

T cell activation by PHA and anti-CD3 antibodies increases GRK expression and activity (40, 41). Hence, we next assessed whether CD46 costimulation could modulate GRK2/3 levels. CD4<sup>+</sup> T cells were activated by immobilized anti-CD3, anti-CD3/CD28 or anti-CD3/CD46 antibodies for 3 days. GRK2/3 levels were then assessed by western-blots using anti-GRK2/3 antibodies. T cell activation by CD28 and especially CD46 led to enhanced levels of GRKs (Figure 1D). Hence, these data demonstrate a relationship between GRKs and the CD46 pathway.

### PGE2 decreases CD46 surface expression in activated T cells only

PGE2 is a known T cell modulator which signals through binding to the E-prostanoid family of GPCRs (namely EP1-EP4). GRK2 regulate expression of the EP4 receptor (24), and we found that EP4 knockdown led to increased CD46 expression (Supp. Figure S1), suggesting a role of PGE2 in controlling CD46 expression. Hence, in order to assess whether signaling through GPCR/GRK could modulate CD46 expression, we next investigated the effect of PGE2 on CD46 expression and function. CD4<sup>+</sup> T cells were activated with anti-CD3, anti-CD3/CD46 or anti-CD3/CD28 antibodies, in presence of PGE2 or DMSO as vehicle control, and expression of CD46 was monitored by flow cytometry. Figure 2A shows the representative data obtained after 2 days of culture for one donor, while Figure 2B shows the average data obtained with the different donors after 2 or 5 days of culture (n=15). The percentage changes in CD46 expression upon addition of PGE2 were also calculated and are presented in Figure 2C. As previously shown, CD46 ligation led a strong downregulation of its expression. A slight increase in CD46 surface expression was detected when CD46 and CD28 were co-ligated, suggesting that there is a crosstalk between CD28 and CD46 that slightly restores CD46 levels (Figure 2B). The addition of PGE2 led to a slight increase in CD46 expression in unstimulated T cells at 2 days (Figure 2B). In contrast, PGE2 treatment led to decreased CD46 expression in activated T cells. While CD3 or CD28 costimulation had only a slight effect on CD46 expression, a stronger reduction (~ 40%) was observed upon CD46 costimulation. The effect of PGE2 was lost in CD3-activated T cells at day 5 (Figure 2B). However, the decrease in CD46 expression was sustained by CD28 and CD46 costimulation, with again the stronger effect observed upon CD46 ligation. Figure 2D shows that the dose-dependent effect of PGE2 on CD46 expression. Hence, PGE2 clearly modulates CD46 expression in activated T cells.



### PGE2 differentially affects T cell activation depending on the costimulation signal

We next assessed the role of PGE2 on T cell activation. CD4<sup>+</sup> T cells were activated with anti-CD3, anti-CD3/CD46 or anti-CD3/CD28 antibodies in presence or absence of PGE2. First, T cell proliferation was determined by <sup>3</sup>H-thymidine incorporation (n=10). As expected, addition of PGE2 significantly decreased T cell proliferation in a dose dependent manner, but the strongest effect was observed upon CD46 costimulation (Figure 3A and 3B). Similar effect was observed when proliferation was determined by CFSE labeling (data not shown). Second, we determined whether the levels of cytokine produced by activated T cells were modified by addition of PGE2. We focused on IL-10 and IFN $\gamma$  as CD46 has the ability to switch T cells from producing IFN $\gamma$  to secreting IL-10 (8). Secretion of IL-10 and IFN $\gamma$  was quantified by ELISA, and we also calculated the IL-10:IFN $\gamma$  ratio to bypass the variations due to the changes in proliferation (n=15). As previously described, CD46 activation promotes a Tr1-like phenotype, visualized by an increase in the IL-10:IFN $\gamma$  ratio (Figure 3C). PGE2 drastically decreased cytokine production (Figure 3C). However, PGE2 had different effects on the relative levels of cytokines produced depending on the activation signals received by the T cells. A trend towards an increase in IL-10:IFN $\gamma$  was observed upon CD3 activation alone, reflecting the known effect on PGE2 in the downregulation of IFN $\gamma$ . Costimulation with CD28 had no significant effect on the IL-10:IFN $\gamma$  ratio, suggesting that the lower level of cytokine production was mainly correlated with the lower proliferation of the cells. In contrast, ligation of CD46 resulted in a significant decrease in the IL-10:IFN $\gamma$  ratio in presence of PGE2. Moreover, the effects of CD46 were dominant over CD28 as also observed upon CD3/CD28/CD46 coligation. Overall, these data indicate that PGE2 exerts different effects on cytokine production that mainly depend on the signals received by T cells.

### PGE2 modulates the phenotype of CD46-costimulated T cells

We next determined whether PGE2 was affecting the phenotype of CD46-costimulated T cells. As PGE2 is known to reduce CD25 expression in CD3-activated T cells (36), we assessed the expression levels of CD25 as a control. We also determined the levels of CD69, another activation marker (Figure 4A). Moreover, as PGE2 has been reported to induce Tregs (36), we determined the expression levels of CTLA-4 (Figure 4A), Foxp3 and CD46 (Figure 4B). Figure 4C represents the average change in expression upon addition of PGE2 obtained for the different donors (n=6). A strong decrease in CD25 expression was detected for all conditions of activation, including CD46-costimulated T cells. Surprisingly, while CD69 expression was strongly decreased in the presence of PGE2 upon CD3 and CD28 activation, its expression was maintained when the cells were costimulated by CD46. T cell activation in the presence of PGE2 also resulted in a decrease in CTLA-4 expression, which was slightly restored upon costimulation. Lastly, PGE2 increased Foxp3 expression in both CD28 and CD46 costimulated T cells. Interestingly, the cells acquiring Foxp3 maintained CD46 expression although CD46 expression in CD46-costimulated T cells was downregulated by PGE2 (Figure 4B). These data show that, depending on the activation signals, PGE2 differentially affects a variety of phenotypic markers in T cells that are involved in regulating T cell activation as well as Treg functions.

### PGE2 inhibits STAT3 phosphorylation

PGE2 decreased CD46 expression in activated T cells, and it has been shown to modulate STAT3 signaling (42, 43). As the CD46 promoter includes a STAT3 binding site and the direct interaction between STAT3 and CD46 has been previously demonstrated by ChIP assays, mutations of the STAT3 binding sites in CD46, and use of anti-STAT3 oligonucleotides (44, 45), we determined the levels of STAT3 phosphorylation in activated T cells. T cells were activated for 24 hrs in the presence or absence of PGE2 and STAT3 phosphorylation determined by flow cytometry. An increase in phosphorylation was

observed in all activated T cells, including CD46-costimulated T cells. Addition of PGE2 decreased STAT3 phosphorylation, with the strongest effect observed upon CD46 ligation (Figure 5).

### Specific changes in PGE2 receptors are induced by CD46 activation

PGE2 binds to four GPCRs, named EP1, EP2, EP3 and EP4. We determined their mRNA expression by qPCR upon T cell activation with anti-CD3, anti-CD3/CD28, anti-CD3/CD46 antibodies for 48hrs (n=3 different donors and experiments performed at different times). As previously reported (33), only EP2 and EP4 were strongly expressed in T cells. Interestingly, CD46 was able to differentially modulate PGE2 receptors compared to CD28 costimulation (Figure 6A). While CD3 and CD28 costimulation induced both EP2 and EP4 expression, CD46 activation only led to an increased EP4 expression, hence strongly increasing the EP4:EP2 ratio (Figure 6B).

To understand the role of EP4 in CD46-activation, we added the selective EP4 antagonist ONO-AE2-227 to T cell culture and assessed cytokine production. Blocking EP4 led to an increase in the IL-10:IFN $\gamma$  ratio, but only when CD46 was ligated, and we did not observe any effect of the EP2 antagonist AH6809 (Figure 6C). This further demonstrates the role of the PGE2:EP4 axis in regulating IL-10 production by CD46-activated T cells.

## DISCUSSION

This study reports novel elements in the complex regulation of primary human T cell activation. Our data show for the first time that the PGE2-EP4-GRK axis is involved in the CD46 pathway and that it modulates CD46 expression and functions. Knocking-down GRKs led to a strong downregulation of CD46 at the surface of activated T cells. It is known that GRK levels are modulated by T cell activation (40, 41), and we report here that CD46 costimulation strongly increased GRK levels expression. Interestingly, decreased levels of GRK2 and GRK6 were observed in T cells from patients with rheumatoid arthritis (46) and MS (47, 48), and proinflammatory cytokines and oxygen radicals can decrease GRK2 levels *in vitro* (49). Hence, the levels of GRKs are highly modulated during inflammation, and this suggests the subsequent modulation of expression of CD46, a key regulator of T cell activation, in inflammatory settings. As CD46 is dysfunctional in T cells from patients with MS and RA, future investigation on the role of GRKs upon CD46 activation in these patients might highlight the molecular mechanisms for this defective pathway.

GRKs control GPCR signaling by phosphorylating these receptors, which provokes their internalization and degradation, a crucial feature of the plasticity of the immune system involved in the regulation of inflammation (reviewed in (23)). Indeed, almost 30% of drugs on the market target GPCR (50, 51). We investigated the role of PGE2, that signals through four subtypes of the E-prostanoid subfamily of GPCRs termed EP1, EP2, EP3 and EP4, in T cell responses. Although the anti-proliferative role of PGE2 on T cells is well established, the role of PGE2 in T cell differentiation is much more complex and not well understood, as it has some complex proinflammatory and immunoregulatory properties. Herein, we have compared the effects of PGE2 in CD28 and CD46 costimulated T cells. As previously shown, PGE2 exerted a strong anti-proliferative role on all activated T cells. However, the effect was much stronger in CD46-costimulated T cells than in CD28-coactivated T cells. Despite the decrease in proliferation, activated T cells did secrete some significant levels of IL-10 and IFN $\gamma$ . In a number of studies, the addition of PGE2 caused a marked reduction in Th1 cytokines (26, 27), while other studies indicate the role of PGE2 in inducing Th1 or Th17 differentiation (28, 33). We found that the relative levels of IL-10 and IFN $\gamma$  secreted were differentially modulated depending on the activation signals received by the T cells. While CD3 activation favored IL-10:IFN $\gamma$ , as previously described, we did not find any



significant effects on CD28-costimulated T cells. However, there was a significant decrease in the IL-10:IFN $\gamma$  ratio upon CD46 ligation. Importantly, similar trends were obtained when naïve CD4<sup>+</sup> T cells were used (data not shown). PGE2 therefore skews the usually regulatory cells towards a more inflammatory secretion profile. PGE2 downregulates both IL-2 and CD25 (26). As IL-2 is required for CD46-mediated Tr1 differentiation (8), it is likely that the effect of PGE2 on IL-2 and CD25 expression as well as the inhibition of CD46 expression contribute to inhibit IL-10 production by CD46-activated T cells. Our data also underline that the actions of PGE2 are largely dependent on the activation of the T cells. Various effects of PGE2 have previously been reported on the secretion of IL-3 by T cells depending on the activation of the cells (52). Hence, it becomes clear that PGE2 differentially modulates T cell responses depending on the environment and signals perceived by the T cells. Our data highlight the role of PGE2 in the production of IFN $\gamma$  and IL-10, which are, at least in part, determined by the regulation of expression of CD46.

PGE2 strongly modulates the phenotype of activated T cells. We show that the addition of PGE2 decreased CD46 expression. This was observed in all activated T cells, although the effect was much more pronounced upon CD46 ligation. Of note, we observed a slight increase in CD46 expression in unactivated T cells. Interestingly, PGE2 has been found to upregulate CD55, another regulator of complement activation molecule, in colon cancer cells (53). Hence, there is a similar pattern for CD46 in resting T cells. However, the increased expression of CD55 might also be to partly counteract the downregulation of CD46 expression observed in activated cells, in order to protect cells from complement attack. Importantly, as CD46 is ubiquitously expressed, it is likely that PGE2 will also modulate its expression and function in other cell types, such as dendritic cells especially as PGE2, like CD46, has been shown to regulate IL-23 production (35, 54).

It was previously demonstrated that PGE2 decreased CD25 expression and upregulated Foxp3 levels in human T cells (36). We confirmed these findings, and report that similar decreased CD25 and increased Foxp3 expression are also detected after CD46 costimulation. Moreover, PGE2 caused a strong reduction of expression of the co-inhibitory molecule CTLA-4 on CD3-activated cells, but CTLA-4 expression was mostly maintained upon costimulation. CTLA-4 is expressed at high levels by Treg cells and is involved in their function (55), hence the increase in Foxp3 correlates with CTLA-4 expression. PGE2 also decreased CD69 expression in activated T cells, although not in CD46-costimulated cells. CD69 is transiently induced after activation, but can persist on leukocyte infiltrates in chronic inflammatory diseases. CD69 deficient mice developed exacerbated forms of arthritis, allergic asthma and other inflammatory settings, and it was proposed that CD69 could act as a regulator of Th17 differentiation (56). The fact that PGE2 maintained CD69 in CD46-costimulated T cells is intriguing and warrants further investigation on this pathway, notably in chronic inflammatory diseases where the CD46 pathway is dysfunctional.

The different effects on cytokine production and phenotype observed in presence of PGE2 were correlated with distinct profiles of expression of PGE2 receptors. CD3 and CD28 led to similar increase in EP2 and EP4, which is consistent to what had been shown in the mouse (57) and in human T cells (33). However, CD46 costimulation largely favored EP4 expression, and addition of an EP4 antagonist increased the IL-10:IFN $\gamma$  ratio by CD46-activated T cells, highlighting the specific role of EP4 in CD46 function. Contrasting effects of PGE2 on cytokine production have previously been shown to be mediated by specific receptors. Boniface *et al.* show that PGE2 can act on CD3/CD28/CD2-coactivated human T cells to promote IL-17, which is mainly mediated by EP2, while it inhibits IFN $\gamma$  and IL-10 production mainly through EP4 signaling (33). Both the EP2 and the EP4 receptors couple with the G $\alpha$ (s) protein whereas the EP1 and the EP3 receptors are known to couple to G $\alpha$ (q) and G $\alpha$ (i), respectively. However, differences in signaling between EP2 and

EP4 have indeed been demonstrated, as for example EP4 but not EP2 leads to ERK phosphorylation via PI3K activation (58). It is likely that different signaling cascades are involved in the CD28 and CD46 pathways. The link between CD46 and EP4 is also intriguing as there is a special emphasis on these receptors in MS and other inflammatory diseases. Among PGE2 receptor-deficient mice, only the EP4-subtype knockout can significantly inhibit experimental autoimmune encephalomyelitis (EAE) (29). Moreover, MS-associated SNPs in EP4 (also called PTEGR4) have been demonstrated (59). As it is now clearly established that the CD46 pathway is dysfunctional in MS (13–16), further investigations should address the question as to whether the CD46-PGE2-EP4-GRK pathway is dysfunctional in MS.

Hence, we propose a model in which PGE2, secreted at sites of inflammation and possibly by T cells themselves (60, 61), binds to EP4, the specific PGE2 receptor induced by CD46 activation. This initiates a signaling cascade that results in decreasing CD46 expression, at least in part through STAT3 inhibition. GRKs are also induced by CD46 costimulation, and will then bind to EP4 and terminate response through internalization of the PGE2 receptor. Of note, while EP4 couples via arrestin upon ligand binding, EP2 does not and is resistant to agonist-induced desensitization (62). Therefore, it seems logical that CD46 would favor EP4 that can be desensitized in order to ensure that proper levels of CD46 expression are restored. When GRKs are knocked-down, the PGE2-EP4 signaling goes on resulting in a continuous decrease in CD46 expression.

The effects of PGE2 are known to be complex, depending on the site and timing of release, and the cell on which it acts. Our data demonstrate the novel role of the PGE2-EP4-GRK axis in CD46 functions, and how this influences the effects of PGE2 on cytokine production and cell surface expression of several markers involved in T cell regulation. The many complex and interlinked actions of PGE2 on lymphocytes, as well as other immune cells will require further investigation to fully decipher the complexity of this system.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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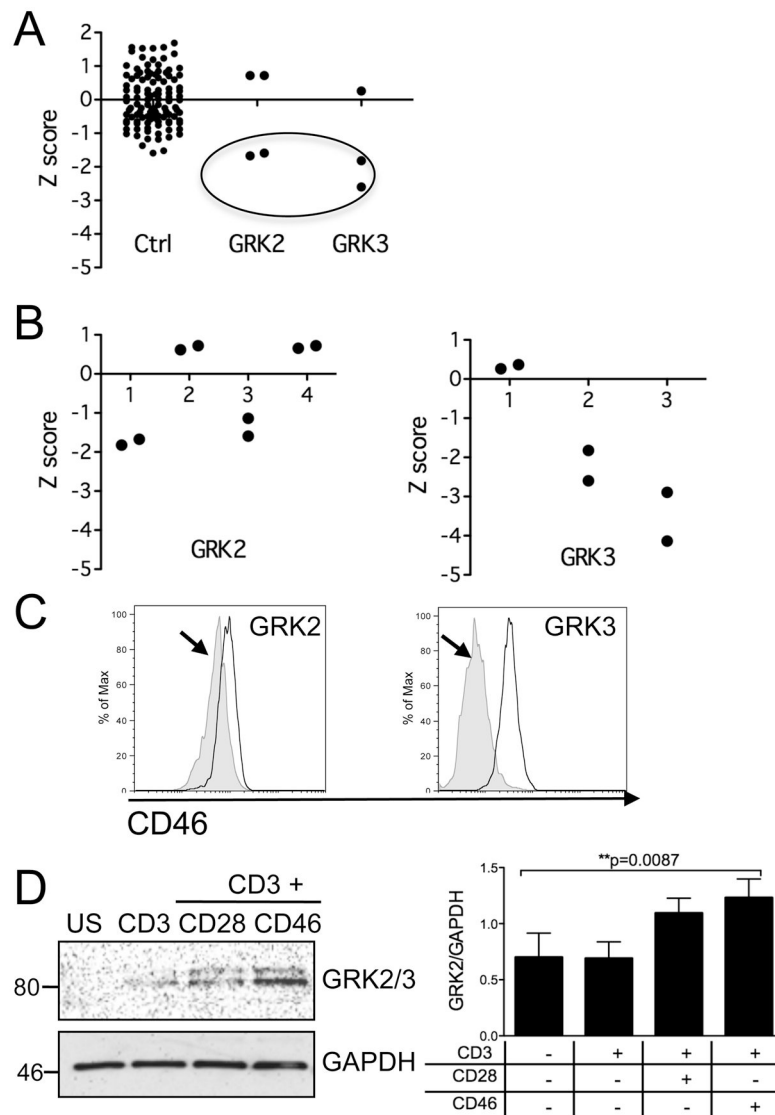
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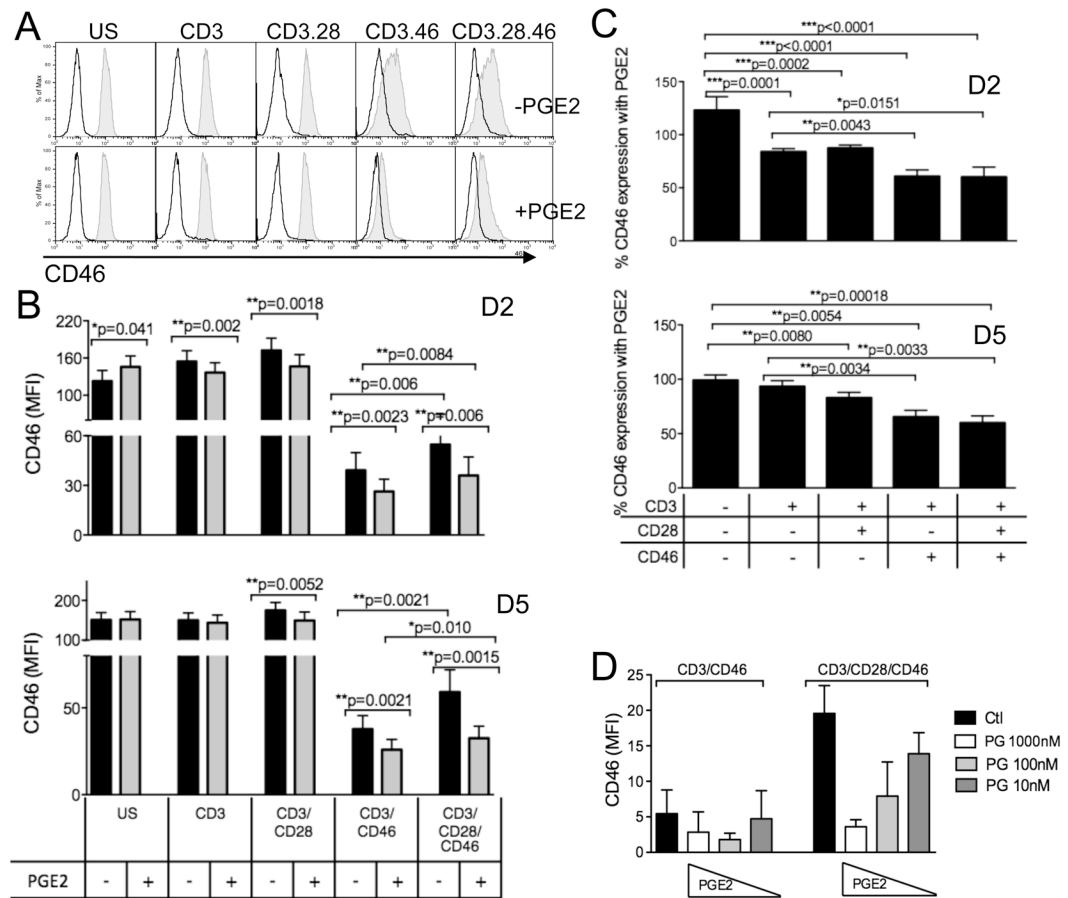


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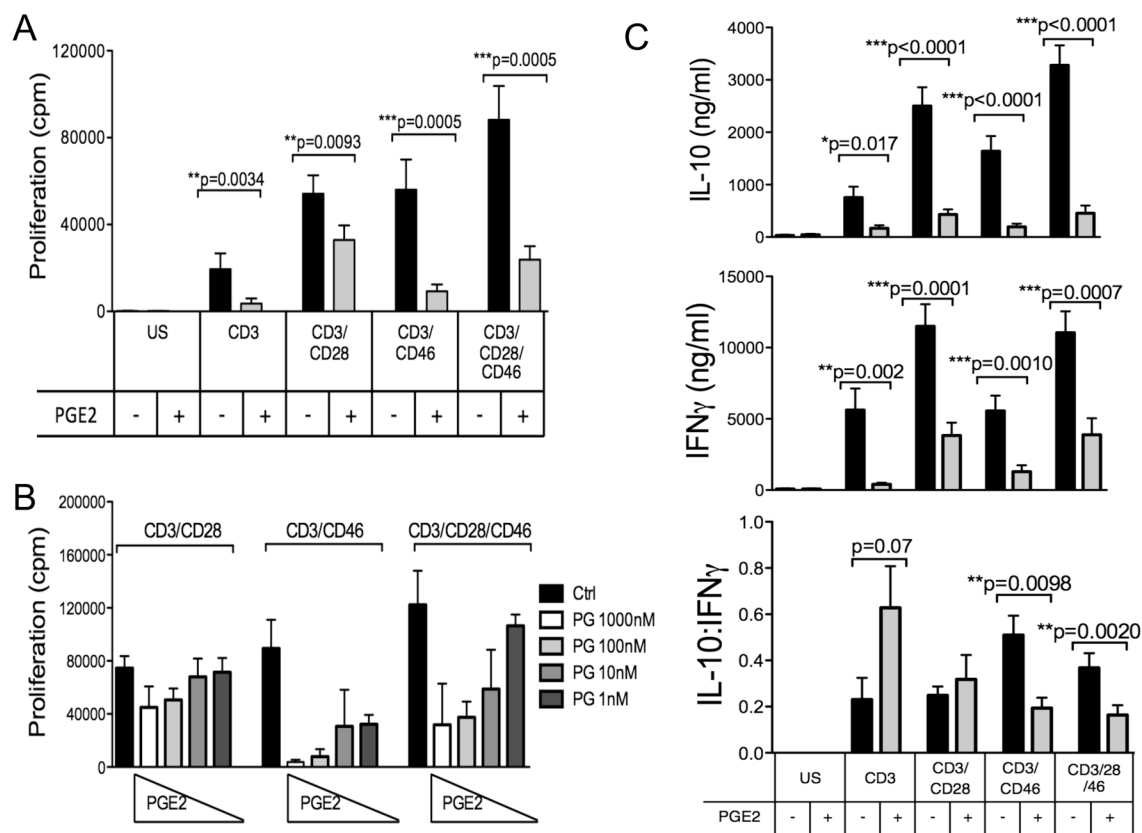
**Figure 1. GRKs regulate CD46 expression in primary T cells**

(A) An RNAi screen was performed on primary CD4<sup>+</sup> T cells infected by lentivirus coding for 5000 different shRNA (1 shRNA construct per well). The Z-score obtained after knockdown of GRK2 and GRK3 compared to controls is shown. Infection by 2 different shRNA constructs targeting GRK2 and GRK3 led to strong negative Z scores, corresponding to a decrease in CD46 expression (circled). (B) The Z-scores obtained across two independent experiments with different donors for the multiple shRNAs targeting GRK2 and GRK3 are shown. The phenotypes observed were repeatedly due to the same hairpins. (C) CD46 expression measured by flow cytometry on cells infected with control lentiviruses or shRNA for GRK2 and GRK3. Arrows point to the constructs inducing CD46 downregulation. (D) CD46 activation induces GRK2/3. CD4<sup>+</sup> T cells were left unstimulated (US) or were activated with immobilized anti-CD3, anti-CD3/anti-CD28, or anti-CD3/CD46 antibodies for 2 days. Cell lysates were obtained and GRK2/3 levels determined by western-blot. Quantification was obtained after stripping of the membrane and reblotting with GAPDH antibodies. The GRK/GAPDH ratio obtained for 4 donors is also represented.



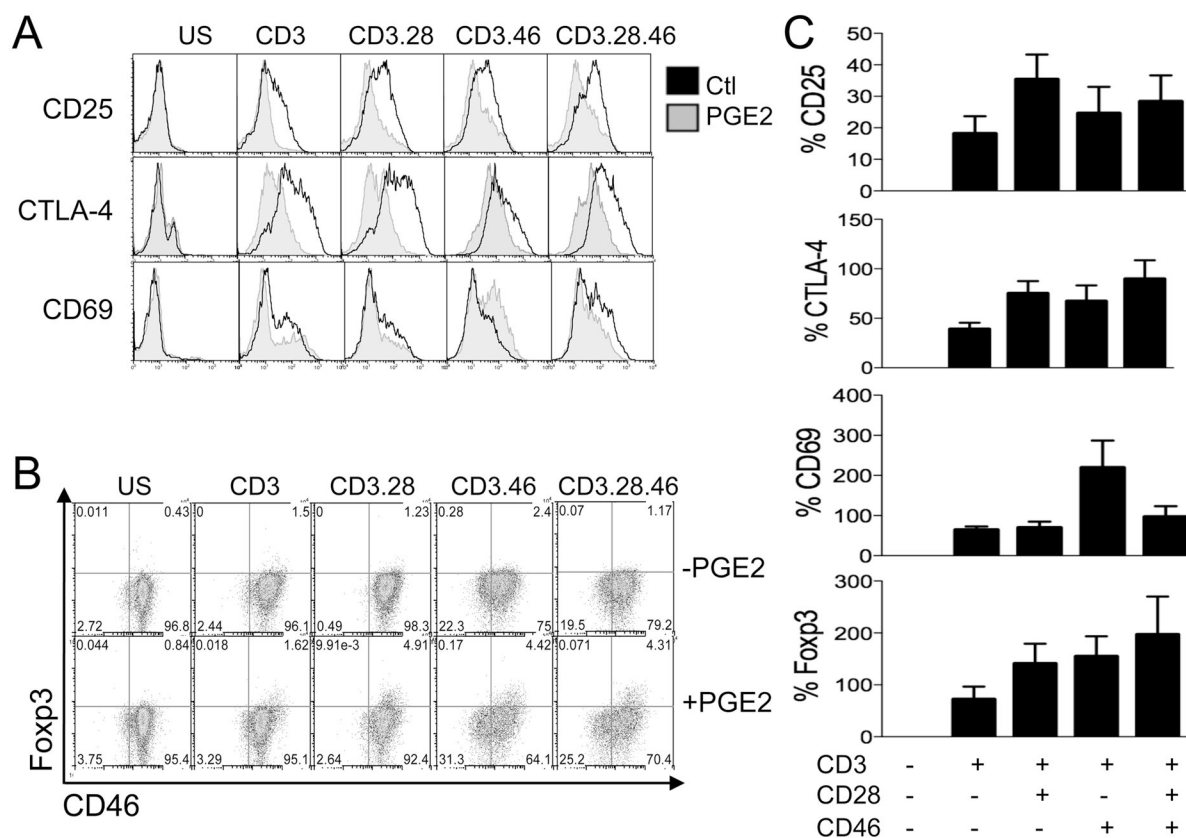
**Figure 2. Prostaglandin E2 regulates CD46 expression**

(A) Purified CD4<sup>+</sup> T cells were left unstimulated (US) or stimulated as indicated by immobilized antibodies in presence of PGE<sub>2</sub> or DMSO as vehicle control. CD46 expression was then monitored by flow cytometry after 2 days (CD46 staining: shaded grey areas; isotype control: black lines). (B) The expression of CD46 was monitored in T cells cultured for either 2 or 5 days in presence or absence of PGE<sub>2</sub> (n=15). (C) The percentage changes in CD46 expression upon addition of PGE<sub>2</sub> are represented. Samples were analyzed using the Wilcoxon test. The means  $\pm$  SEM are shown (n=15 donors). (D) The expression of CD46 was monitored in T cells cultured for 3 days in presence of increasing doses of PGE<sub>2</sub>, as indicated (n=3).



**Figure 3. PGE2 modulates T cell activation depending on the activation signals**

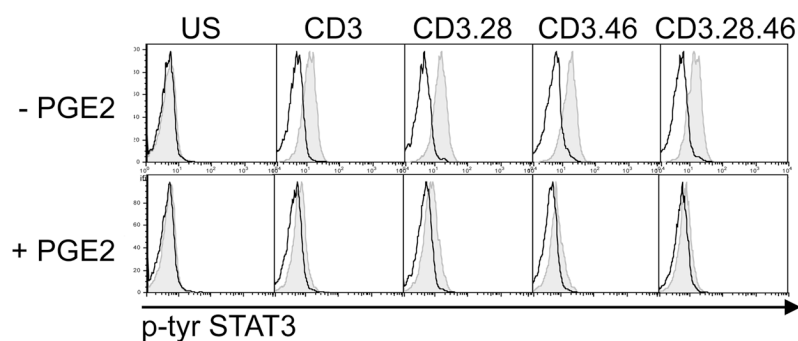
Purified CD4<sup>+</sup> T cells were stimulated by immobilized antibodies as indicated in presence or absence of PGE2. **(A)** Proliferation was determined after 4 days by <sup>3</sup>H incorporation (n=10). **(B)** The dose effect of PGE2 on T cell proliferation of T cells activated by anti-CD3/CD28, anti-CD3/CD46 or anti-CD3/CD28/CD46 is shown (n=3). **(C)** Cells were activated as in (A) and the amounts of IL-10 and IFNγ in the supernatants were determined by ELISA (n=15). The IL-10:IFNγ ratio is also represented to show specific effects independent of the changes in proliferation induced by PGE2.



**Figure 4. PGE2 modulates T cell phenotype upon activation**

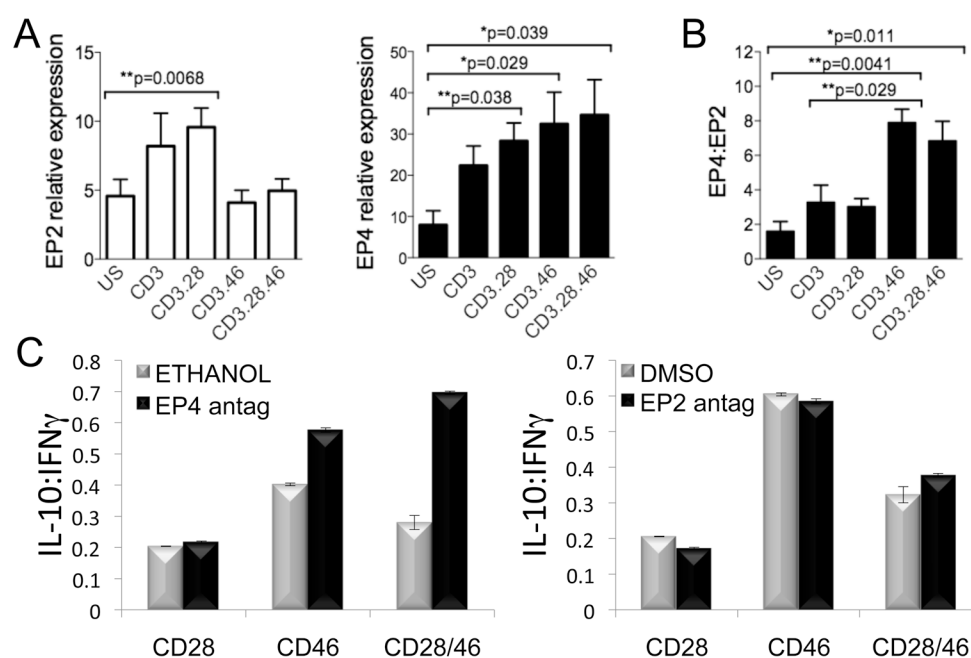
(A) Purified CD4<sup>+</sup> T cells were stimulated with immobilized anti-CD3, anti-CD3/anti-CD28 (CD3.28), anti-CD3/CD46 (CD3.46) or anti-CD3/CD28/CD46 (CD3.28.46) antibodies in presence (shaded grey histograms) or absence (black line) of PGE2. After 5 days, the expression of surface CD25 and CD69 and intracellular CTLA-4 was determined by flow cytometry. (B) Expression of Foxp3 and CD46 was also determined after intracellular staining. (C) The percentage changes in CD25, CTLA-4, CD69 and Foxp3 expression upon addition of PGE2 obtained for the different donors are represented (n=6).





**Figure 5. PGE2 inhibits STAT3 phosphorylation**

Purified CD4<sup>+</sup> T cells were stimulated by immobilized antibodies as indicated in presence or absence of PGE2. After 24 hrs, the level of p-Tyr STAT3 was determined by flow cytometry (representative of two independent experiments). (STAT3 staining: shaded grey areas; isotype control: black lines).



**Figure 6. T cell activation results in expression of different PGE2 receptors depending on the activation signal**

Purified CD4<sup>+</sup> T cells were stimulated by immobilized antibodies as indicated, for 2 days. mRNA were then prepared and the expression levels of the 4 PGE2 receptors (EP1-4) determined. **(A)** Relative expression of EP2 and EP4 in activated T cells. The means  $\pm$  SEM are shown (n=3 donors). Samples were analyzed using the Wilcoxon test. **(B)** The ratio of EP4:EP2 relative expressions has been plotted. CD46 activation results in an increased EP4:EP2 ratio. **(C)** EP4 is involved in the modulation of cytokines produced by CD46-costimulated T cells. Purified CD4<sup>+</sup> T cells were stimulated by immobilized antibodies as indicated in presence of EP2 or EP4 antagonist, and DMSO or ethanol as a control, respectively. The levels of IL-10 and IFN $\gamma$  were quantified and the IL-10:IFN $\gamma$  ratio is represented. Representative of 3 experiments.